EXHIBIT A

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Structural and Functional Roles of Asparagin 175 in the Cysteine Protease Papain*

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The role of the asparagine residue in the Cys-His-Asn "catalytic triad" of cysteine proteases has been investigated by replacing Asn^{175} in papain by alanine and glutamine using site-directed mutagenesis. The mutants were expressed in yeast and kinetic parameters determined against the substrate carbobenzoxy-L-phenylalanyl-(7-amino-4-methylcoumarinyl)-L-arginine. At the optimal pH of 6.5, the specificity constant $(k_{cat}/K_m)^{obs}$ was reduced by factors of 3.4 and 150 for the $Asn^{175} \rightarrow Gln$ and $Asn^{175} \rightarrow Ala$ mutants, respectively. Most of this effect was the result of a decrease in k_{cat}, as neither mutation significantly affected K_m. Substrate hydrolysis by these mutants is still much faster than the non-catalytic rate, and therefore Asn^{175} cannot be considered as an essential catalytic residue in the cysteine protease papain. Detailed analyses of the pH activity profiles for both mutants allow the evaluation of the role of the Asn^{175} side chain on the stability of the active site ion pair and on the intrinsic activity of the enzyme. Alteration of the side chain at position 175 was also found to increase aggregation and proteolytic susceptibility of the proenzyme and to affect the thermal stability of the mature enzyme, reflecting a contribution of the asparagine residue to the structural integrity of papain. The strict conservation of Asn^{175} in cysteine proteases might therefore result from a combination of functional and structural constraints.

Cysteine proteases are a class of enzymes requiring the thiol group of a cysteine residue for their catalytic activity (1). The additional involvement of an histidine residue in the catalytic process was inferred on kinetic grounds (2), and evidence for the location of an histidine in proximity to the catalytic thiol group was provided initially by the use of a bifunctional irreversible inhibitor of papain (3). The Cys^{25}-His^{159} arrangement in the catalytic center of papain was established when the three-dimensional structure of the enzyme was solved (4–6). The papain molecule is folded to form two interacting domains delimiting a cleft at the surface of the enzyme. Cys^{25} and His^{159} are located at the interface of this cleft on opposite domains of the molecule; Cys^{25} is part of the L1 α-helix at the surface of the left domain, while His^{159} is in a β-sheet at the surface of the right domain of the enzyme.

With the availability of the three-dimensional structure, other residues were found in the vicinity of the active site that could possibly play important roles in the mechanism of the enzyme. In particular, an asparagine residue that is conserved in all cysteine protease sequences of the papain family, Asn^{175}, was found to be adjacent to the catalytic His^{159} residue. The amide oxygen of the Asn^{175} side chain is hydrogen-bonded to $N^{\delta}H$ of His^{159} creating a Cys-His-Asn triad, which can be considered as being analogous with the Ser-His-Asp triad of serine proteases (Fig. 1). The side chain of Asn^{175} is buried in a hydrophobic region of the enzyme composed mainly of residues Phe^{141}, Val^{161}, Trp^{177}, and Trp^{181}. Residues 141, 177, and 181 are located near the Asn^{175}-His^{159} hydrogen bond and can shield it from the external solvent. An important feature of the Asn^{175}-His^{159} interaction is that the hydrogen bond is approximately colinear with the His^{159} C^{β}-C^{γ} bond, allowing rotation of the imidazole ring about the C^{β}-C^{γ} bond without disruption of the Asn^{175}-His^{159} hydrogen bond. Comparison of results from crystallographic studies with various forms of papain either free or alkylated at the Cys^{25} sulfur atom by chloromethyl ketone inhibitors have demonstrated that the His^{159} side chain can change its orientation by about 30° (7). Therefore, it has been suggested that the role of Asn^{175} is to orient the His^{159} side chain in the optimum positions for various steps of the catalytic mechanism. In the resting state of the enzyme, the His side chain would be coplanar to the Cys^{25} residue while during acylation, the protonated imidazole ring would rotate to act as a proton donor to the nitrogen atom of the leaving group of the substrate (8).

An important feature of papain and other cysteine proteases in general is the high nucleophilicity of the sulfur atom of the active site cysteine residue. This is due to the fact that at the pH values where the enzyme is active, the sulfur atom is present as a thiolate anion. It is now generally accepted that the side chains of Cys^{25} and His^{159} possess unusual pK_a values and that the active form of the enzyme consists of a thiolate-imidazolium ion pair at neutral pH (9–12). However, the nature and significance of the factors that are responsible for the formation and maintenance of the ion pair within a wide range of pH for the most part remain unknown, and this aspect has been the object of many theoretical studies over the years (see, *e.g.*, Refs. 13–19). Since the side chains of Asn^{175} and His^{159} interact directly via hydrogen bonding, one of the obvious roles of Asn^{175} could be to stabilize the thiolate-imidazolium form of papain. It has been suggested that the proximity of the active site cysteine and histidine residues could be one of the most important factors contributing to the formation of an ion pair and that the proton affinities of Cys^{25} and His^{159} at the active site of papain are strongly sensitive to the geometry of these residues (17, 19). Consequently, Asn^{175} could stabilize the ion

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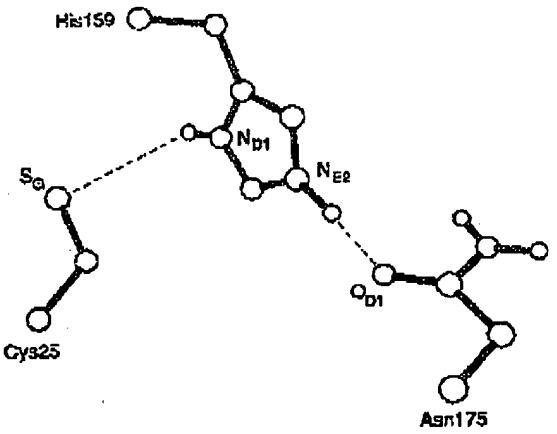

FIG. 1. Schematic representation of the active site of papain showing the catalytic triad residues Cys^{25}, His^{159}, and Asn^{175}. The representation is derived from the crystal structure of Drenth *et al.* (7). In the crystal structure, the active site cysteine is oxidized, and therefore the precise relative orientations of the Cys^{25} and His^{159} side chains in the non-oxidized enzyme might differ from the illustrated orientations.

pair by keeping the imidazole ring of His^{159} in a favorable orientation.

There has been no quantitative experimental study addressing the role of the asparagine residue in the catalytic triad of cysteine proteases. In a preliminary study using random mutagenesis and screening of mutants, we have shown that replacement of Asn^{175} by several amino acids results in a significant loss of activity (20). However, due to the relatively low sensitivity of the assay, this system can unambiguously detect only mutants with activity similar to wild-type papain. In addition, enzyme inactivation could occur for mutant enzymes that have a decreased stability under the relatively drastic conditions used to activate the enzyme precursors (low pH and high temperature). The screening system we used cannot readily distinguish between a decrease in catalytic activity and a decrease in protein stability. In this study, the role of Asn^{175} at the active site of cysteine proteases was investigated by a detailed kinetic and functional characterization of papain mutants. Mutation of Asn^{175} to a glutamine was chosen due to our previous observation that this mutation generates an enzyme that retains some activity (20), indicating that the conservative substitution of Asn^{175} by Gln is tolerated in the active site of papain. Complete removal of the hydrogen bonding capability of the side chain of residue 175 was accomplished by an Asn^{175} → Ala change.

EXPERIMENTAL PROCEDURES

Expression and Purification of Papain Mutants—Expression of wild-type papain and of the Asn^{175} → Gln and Asn^{175} → Ala mutant proenzymes in *Saccharomyces cerevisiae* has been reported recently (20). Yeast cells from 1 liter of culture (8×10^7 cells/ml) were collected by centrifugation and resuspended in 20 ml of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA to yield a final volume of about 35 ml. The cells were lysed using a French press (20,000 p.s.i.) and the cellular debris removed by a 10-min, 15,000 × *g* centrifugation. Propapain present in the supernatant was converted to mature papain by limited proteolysis with subtilisin BPN′ (Sigma). The soluble extract was incubated for 2–3 h at 37 °C in the presence of 0.1 mg/ml subtilisin. The extract was then changed to pH 5.0 with sodium acetate buffer (30 mM, pH 4.0) and incubated at 55 °C for 15 min. After a 10-min centrifugation at 15,000 × *g*, precipitated proteins were discarded and the supernatant was made 80% ammonium sulfate and kept at 4 °C overnight. This suspension was centrifuged at 22,000 × *g* for 20 min and the protein pellet resuspended in 4 ml of 100 mM sodium acetate, 1 mM EDTA, pH 5.8. This preparation was used to determine the protein half-life (see below). The enzymes used for the kinetic characterization were further purified by covalent chromatography using a thiopropyl-Sepharose column (21).

Kinetics of Irreversible Thermal Inactivation—The kinetics of irreversible thermal inactivation of papain variants was determined as described previously (22). Partially purified papain preparations (see above) were adjusted to pH 6.0 with 100 mM phosphate buffer and $HgCl_2$ added to 5 mM. They were incubated at 82 °C for 0–60 min, and the residual papain activity was measured. The TI_{50} value (the time at which the enzyme has lost half of its activity) was determined from the slope of the linearized form of the data (22).

Aggregate/Soluble Precursor Partitioning and Susceptibility to Protease Degradation—Total yeast extracts (3 ml) were prepared from 75 ml of culture grown under the conditions defined above. Processing of propapain was prevented with 0.1 mM of E-64 (1-[[(*L-trans*-epoxysuccinyl)-L-leucyl]amino]-4-guanidinobutane) (23). The extract was deglycosylated by incubation for 2 h at 37 °C in the presence of 35 mM sodium acetate buffer, pH 5.5, 200 mM β-mercaptoethanol, and 50 milliunits/ml endoglycosidase H (Boehringer Mannheim). An aliquot of the mixture was centrifuged at 15,000 × *g* for 5 min. The supernatant was recovered and the pellet was resuspended in 200 μl of phosphate buffer, pH 6.5. An aliquot of the pellet and supernatant deglycosylated fractions was analyzed by Western blot prior to or following incubation with 0.1 mg/ml subtilisin for 2 h at 37 °C. Quantitative Western blot analyses were performed using two rounds of antigen detection after separation of the proteins in SDS-polyacrylamide gel electrophoresis. Mature and proenzyme forms of papain were detected with an anti-papain rabbit polyclonal antibody (24). Papain-antibody complexes were labeled with I^{125}-labeled protein A (Amersham Corp.) and visualized by autoradiography. The antigen was then stained in a second reaction using alkaline phosphatase-conjugated goat anti-rabbit IgGs (Bio-Rad). This procedure facilitates accurate cutting of the immunoreactive bands for radioactivity measurements using an LKB 1282 Compugamma counter.

Kinetic Measurements—The kinetic parameters were obtained as described previously (21). The concentration of active purified enzyme was determined by titration with E-64 (25). Carbobenzoxy-L-phenylalanyl-(7-amino-4-methylcoumarinyl)-L-arginine (Cbz-Phe-Arg-MCA)[1] was used as a substrate. The reaction conditions consisted of 50 mM phosphate buffer, 0.2 M NaCl, 5 mM EDTA, 10% CH_3CN, pH 6.5. For the determination of pH activity profiles, 50 mM citrate or 50 mM borate were also used as buffers and the substrate concentration was kept well below the K_m value. Kinetic parameters at optimum pH (6.5) were determined by linear regression of the initial rate (v) data to plots of s/v *versus* s (Hanes plots). The pH activity profiles were analyzed according to the model of Reaction 1 by nonlinear regression of the data to the corresponding equation (Equation 1).

$$EH_2 \underset{}{\overset{K_1^{obs}}{\rightleftharpoons}} EH \underset{}{\overset{K_2^{obs}}{\rightleftharpoons}} E$$
$$\downarrow (k_{cat}/K_M)^{lim}$$

REACTION 1

$$(k_{cat}/K_M)^{obs} = \frac{(k_{cat}/K_M)^{lim}}{\frac{[H^+]}{K_1^{obs}} + 1 + \frac{K_2^{obs}}{[H^+]}} \qquad \text{(Eq. 1)}$$

In this equation, $(k_{cat}/K_M)^{obs}$ represents the experimentally determined value of the specificity constant and $(k_{cat}/K_M)^{lim}$ is the limiting value determined from nonlinear regression.

Computer Modeling—Computer modeling was used with the Asn^{175} → Gln mutant to predict the orientation of the Gln^{175} side chain. The model representing free papain was obtained using the coordinates from the crystal structure of Drenth *et al.* (7). In the model, the oxygen atoms on the oxidized Cys^{25} residue were removed, and AMBER partial charges were assigned considering that the active site residues are present in the thiolate-imidazolium ion pair state. In an initial step, the Systematic Search module of Sybyl 6.0 (Tripos Associates, Inc.) was used to carry out a search for sterically allowed conformations of the Asn^{175} → Gln mutant. The Asn^{175} residue was replaced by Gln and the side chain angles χ_1, χ_2, and χ_3 of Gln^{175} were varied by 2-degree increments. Two "groups" of structures (structure 1 and structure 2) were found, both containing an hydrogen bond between the oxygen atom of the Gln^{175} side chain amide and $N^{\delta 2}H$ of His^{159}. The structures

[1] The abbreviations used are: Cbz-Phe-Arg-MCA, carbobenzoxy-L-phenylalanyl-(7-amino-4-methylcoumarinyl)-L-arginine; WT, wild-type.

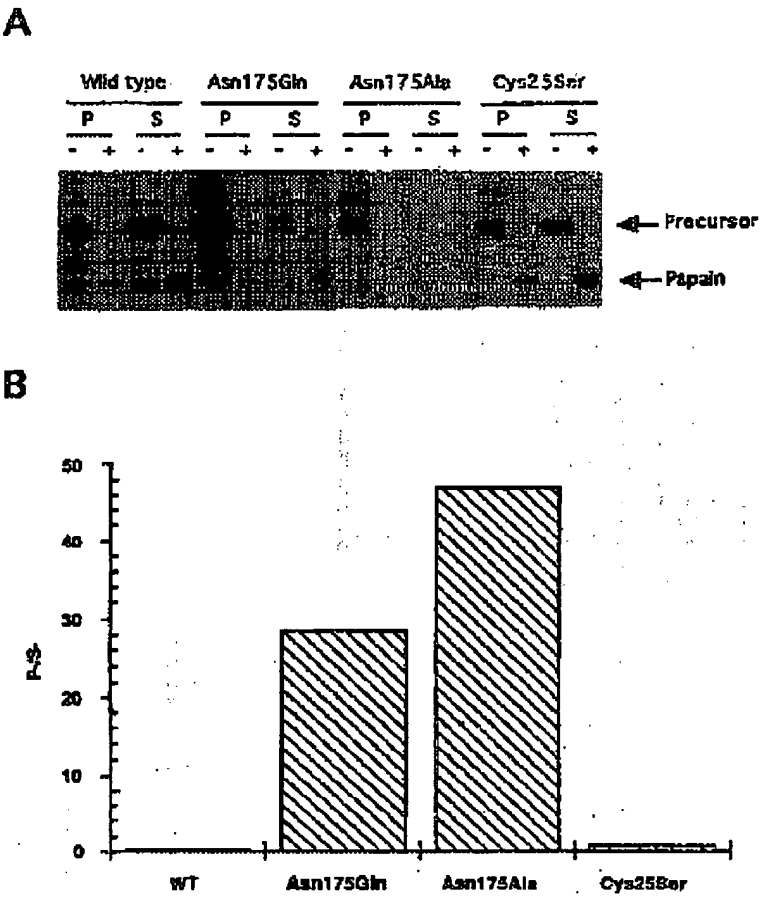

FIG. 2. **Segregation of propapain mutants between pellet and supernatant and sensitivity to proteolytic degradation by subtilisin.** *Panel A*, autoradiogram of Western blot for the pellet fraction (*P*) and the supernatant fraction (*S*) before (−) and after (+) treatment with subtilisin. The source of the sample is indicated above each series of four samples. The location of mature papain and propapain is indicated in the *right margin* of the autoradiogram. *Panel B*, histogram representing the relative levels of insoluble (*P*−) to soluble (*S*−) proenzymes.

differ by the positioning of the acetamide NH_2 group, which can be either in proximity of Trp^{177} and Trp^{181} (structure 1) or oriented toward the interface between the two domains of papain where it can form a hydrogen bond with the side chain of Ser^{176} (structure 2). Each one of these two conformations was energy-minimized in an attempt to predict if one orientation would be favored over the other. Conformational energies were calculated using the AMBER force field and partial charges. A distance-dependent dielectric constant, $\varepsilon = r$, was used with a residue-based cutoff distance of 8.0 Å. The minimization was carried out to a root mean square gradient of 0.05. Energy calculations were performed on a region delimited by a 12-Å sphere around the C^{α} atom of Gln^{175}, while atoms within a 9-Å sphere around the same C^{α} atom were allowed to move during the minimization. The difference in energy between the two minimized structures was 2.0 kcal/mol in favor of structure 1. However, this value is close to the precision of the calculations and the second conformational state (structure 2) cannot clearly be ruled out. For structure 1, the torsional angles of the Gln^{175} side chain are $\phi = -115.77$, $\psi = 160.84$, $\chi_1 = -119.87$, $\chi_2 = -85.88$, and $\chi_3 = 77.61$, and the Gln^{175} $O^{\varepsilon 1}$-His^{159} $N^{\varepsilon 2}H$ distance is 2.77 Å. With this conformation, the acetamide H atoms of Gln^{175} are positioned to interact with the π clouds of the two tryptophan residues (Trp^{177} and Trp^{181}). In the alternate structure (structure 2), $\phi = -116.53$, $\psi = -179.33$, $\chi_1 = -120.38$, $\chi_2 = -67.36$, and $\chi_3 = -92.45$, and the Gln^{175} $O^{\varepsilon 1}$-His^{159} $N^{\varepsilon 2}H$ distance is 2.76 Å.

RESULTS

Pellet/Supernatant Partitioning and Protease Susceptibility of Propapain—We have investigated the consequences of replacing Asn^{175} upon the ability of the protein to be detected as a molecule with native properties. During the course of purifying papain, we observed that the yield of mature papain recovered following *in vitro* trans-activation was much lower for mutants at position 175 than for the wild-type enzyme. Using Western blot analysis, we have shown previously that the total amount of propapain produced was not affected by the mutations (22). Therefore, the differences in yield are not consequences of variations in the transcription or translation efficiency or intracellular instability of the proteins. The reduction in yield, which is more pronounced for the $Asn^{175} \rightarrow Ala$ mutant, could, however, reflect an increased susceptibility to proteolytic degradation by subtilisin in the activation step for the mutants. This suggests that some of the molecules may not be properly folded. Since unfolded proteins are often found to aggregate (26), we have measured the solubility of propapain mutants and the sensitivity of soluble and insoluble fractions to degradation by subtilisin. In the presence of exogenous proteases, the 38-kDa wild-type propapain is fully converted into 24-kDa mature papain (23). This limited proteolytic processing can be easily distinguished from more extensive and less specific degradation of unfolded mutants. A large proportion of propapain mutants at position 175 is found in the pellet fraction (Fig. 2*A*, *lanes P*−), the effect being more pronounced for the $Asn^{175} \rightarrow Ala$ mutant. The pellet fraction is completely degraded by subtilisin (Fig. 2*A*, *lanes P*+), whereas the soluble fraction (Fig. 2*A*, *lanes S*−) is fully converted to mature papain (Fig. 2*A*, *lanes S*+). The ratio of aggregated to soluble fraction is about 0.3 for the wild-type propapain (Fig. 2*B*). This ratio is close to 30 and about 50 for the $Asn^{175} \rightarrow Gln$ and $Asn^{175} \rightarrow Ala$ mutants, respectively. The $Cys^{25} \rightarrow Ser$ mutant was used as a control and has a behavior similar to that of wild-type propa-

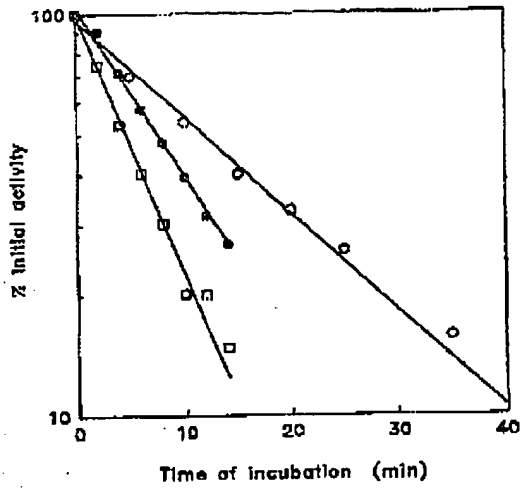

FIG. 3. Kinetics of thermal inactivation of mature papain. Partially purified preparations of WT (○), Asn^{175} → Gln (●), and Asn^{175} → Ala (□) mature papain were incubated at 82 °C for various periods of time under conditions described under "Experimental Procedures," and the level of remaining activity was measured. Each point is the average value of three (Asn^{175} mutants) or two (wild-type) independent measurements.

pain (Fig. 2, *A* and *B*). Thus, the partitioning of propapain between soluble, protease-resistant fractions and insoluble, protease-susceptible fractions is markedly altered by the replacement of Asn^{175}.

The previous results suggest that mutation at position 175 has a detrimental effect upon the ability of the proenzyme to fold in the cell but that, when the protein is folded, it becomes resistant to proteolytic degradation. However, subtilisin can selectively remove the pro region of the precursor and release mature active papain. To determine if mutations at position 175 affect the stability of mature papain, we have measured the rate of thermal inactivation of papain mutants as defined previously (22). The mature enzyme is known to be highly stable to thermal inactivation, as shown by the half-life of 12.5 ± 0.9 min measured for wild-type papain at 82 °C. For the Asn^{175} → Gln and Asn^{175} → Ala mutants, the half-life times at 82 °C are 7.3 ± 0.6 min and 4.6 ± 0.3 min, respectively (Fig. 3), indicating that the mutations also have an effect on the thermal stability of the mature enzyme.

Kinetic Characterization—The papain mutants Asn^{175} → Gln and Asn^{175} → Ala used for kinetic characterization were purified by covalent affinity chromatography. The kinetic parameters at optimum pH (6.5) for hydrolysis of Cbz-Phe-Arg-MCA by the Asn^{175} mutants and wild-type papain are given in Table I. Removal of the Asn^{175} side chain by replacing asparagine by an alanine residue leads to a marked 150-fold decrease in $(k_{cat}/K_M)^{obs}$ at pH 6.5. This effect on activity can be entirely attributed to a decrease in k_{cat}, which is 0.28 s^{-1} for the mutant Asn^{175} → Ala as compared to 41.6 s^{-1} for wild-type papain. Mutation of residue 175 to an alanine therefore has a marked effect on the activity of papain. However, if the Asn^{175} residue is replaced by a glutamine, the kinetic parameters for the mutant show relatively little deviation from those of wild-type. The $(k_{cat}/K_M)^{obs}$ value for Asn^{175} → Gln is 135 × 10^3 M^{-1} s^{-1}, a value only 3.4-fold lower than that of wild-type enzyme. Replacing an Asn by a Gln can be considered as an insertion of an extra methylene group in the side chain of the Asn residue, and the enzyme seems to be able to tolerate this modification as determined by the kinetic properties of the enzyme.

The influence of pH on $(k_{cat}/K_M)^{obs}$ for the Asn^{175} → Gln and Asn^{175} → Ala mutants is illustrated in Fig. 4 (*A* and *B*, respectively). The pH activity profiles of the mutants are significantly narrower than that of wild-type papain (represented by a *dashed line* in the figures), particularly in the case of the Asn^{175} → Ala mutant. Once again, replacement of Asn^{175} by an alanine has a more pronounced effect than mutation to a glutamine. The pH activity profiles of the mutant enzymes can be fitted to an equation describing a model where two pK_a values (*i.e.* two ionizable groups) are considered, one for each limb of the bell-shaped profile. For the wild-type enzyme, the profile is best described by a three-pK_a model, the additional ionizable group influencing the activity of the enzyme only in the low pH region (21). Due to the precision of our experimental measurements with the mutant enzymes, we cannot rule out the possibility that a third ionizable group also modulates the activity in the acid limb of the pH activity profiles for the mutants. However, this group would have only a small effect on activity, as observed with wild-type enzyme (21). In addition, since the low pH limb of the profile for the mutant enzymes is displaced to higher pH values, the third ionizable group might not modulate the activity in the pH range where the Asn^{175} → Ala and Asn^{175} → Gln mutants are active. The value of pK_1^{obs}, which is 4.54 for wild-type papain (see Table I) increases to 5.42 for Asn^{175} → Ala. Similarly, pK_2^{obs} is seen to decrease significantly from 8.45 in wild-type papain to 7.75 in the Asn^{175} → Ala variant. These variations in the pK_a values of the ionizable groups that modulate the activity of papain are the largest observed so far with mutants of this enzyme.

Considerations on the Stability of the Thiolate-Imidazolium Form of Papain—It is generally accepted that the active form of papain consists of a thiolate-imidazolium ion pair (9–12). The stability of this ion pair is considered to be very sensitive to its environment. In the present study, a perturbation of the ion pair is a likely possibility since Asn^{175} interacts directly with one of its members. The O$^{\delta1}$ atom of Asn^{175} is hydrogen-bonded to N$^{\epsilon2}$ of His^{159}, and this interaction could be important for stabilization of the thiolate-imidazolium ion pair form of the active site residues in cysteine proteases. It has been shown previously that any factor influencing the ion pair stability will consequently have an effect on the observed activity ($(k_{cat}/K_M)^{obs}$) of the enzyme (27). In the same study, it was also shown that using certain assumptions, the effect of a mutation on ion pair stability and on the intrinsic activity (k_{cat}/K_M) of papain can be dissected out by a detailed analysis of the pH activity profile.

The model introduced to establish the relationship between the stability of the thiolate-imidazolium ion pair and the measured kinetic parameters has not been applied so far to the characterization of mutations involving directly one of the active site residues (Cys^{25}, His^{159}, or Asn^{175}). To be applicable to the analysis of mutations at position 175 of papain, the equations deduced from the model need to be expanded. In its simplest form, the model describing the ionization pathways of the active site residues is represented in Fig. 5. The four protonation states of the active site residues are considered, and K_R is an equilibrium constant used to describe the conversion of the neutral form (−SH, −Im) to the ion pair form (−S$^-$, −ImH$^+$) of these residues. In the previous study (27), equations were derived assuming that the difference between pK_1 and pK_R, the intrinsic pK_a values for the ionization of Cys^{25} and His^{159} in absence of factors stabilizing the ion pair, is the same for both wild-type and mutant enzymes. In the present study, this condition is not necessarily met and a more general equation has to be introduced (Equation 2),

$$\Delta\Delta pK = 2 \cdot \log\left(\frac{K_{mut} + 1}{K_{wt} + 1}\right) + \Delta pK_a - \Delta pK_R \qquad \text{(Eq. 2)}$$

TABLE I
Kinetic parameters for hydrolysis of Cbz-Phe-Arg-MCA by papain variants

Enzyme	k_{cat}	K_M	$(k_{cat}/K_M)^{lim}$	pK_1^{obs}	pK_2^{obs}	$\Delta\Delta pK$	$(k_{cat}/K_M)^{lim}$
	s^{-1}	mM	$10^{-3} \times M^{-1} s^{-1}$				$10^{-3} \times M^{-1} s^{-1}$
Wild-type[a]	41.6 ± 6.8	0.089 ± 0.006	464 ± 44	4.54 ± 0.29	8.45 ± 0.02		482 ± 46
Asn^{175} → Ala	0.38 ± 0.15	0.124 ± 0.042	3.08 ± 0.80	5.42 ± 0.21	7.75 ± 0.19	−1.58	3.6 ± 0.9
Asn^{175} → Gln	18.9 ± 4.5	0.146 ± 0.062	135 ± 27	5.02 ± 0.04	7.86 ± 0.16	−1.07	145 ± 29

[a] For wild-type papain, the pH activity profile in the acid limb is best described by two pK_a values of 3.6 and 4.54 (40). The highest of the two pK_a values is considered to represent ionization of the same group as the one modulating the activity for the mutant enzymes and was therefore assigned to pK_1^{obs} in the table (see text).

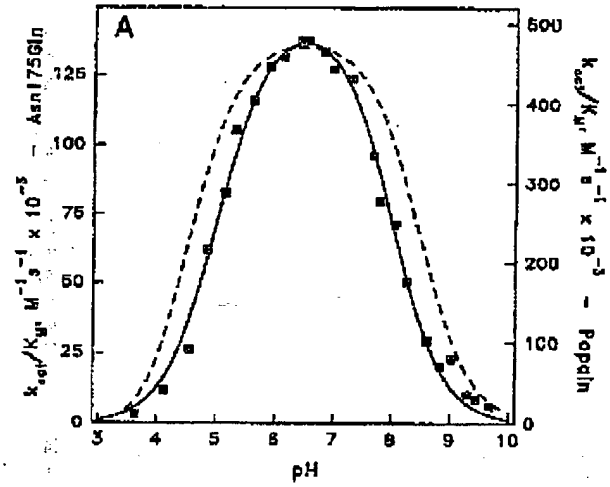

FIG. 4. **pH dependence of $(k_{cat}/K_M)^{obs}$ for the Asn^{175} → Gln (*panel A*) and Asn^{175} → Ala (*panel B*) mutants.** The *solid line* represents the best fit to Reaction 1, obtained by nonlinear regression of the data to Equation 1. The corresponding pH activity profile for wild-type papain is included for comparison (*dashed line*).

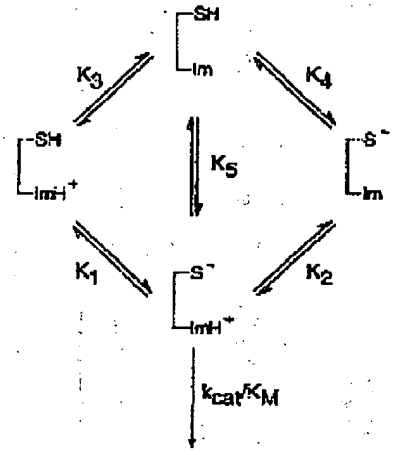

FIG. 5. **Model describing the ionization pathways for the active site residues Cys^{25} and His^{159} of papain and cysteine proteases in general.**

where $\Delta\Delta pK = (pK_2^{obs} - pK_1^{obs})_{mut} - (pK_2^{obs} - pK_1^{obs})_{WT}$, the variation in width of the pH activity profile on going from wild-type papain to the mutant enzyme; $\Delta pK_3 = (pK_{3mut} - pK_{3WT})$ and $\Delta pK_4 = (pK_{4mut} - pK_{4WT})$ are the variations in the intrinsic pK_a values, pK_3 and pK_4, resulting from the mutation; K_{5mut} and K_{5WT} represent the equilibrium constant K_5 for the mutant and wild-type enzymes, respectively. Since the value of $(\Delta pK_4 - \Delta pK_3)$ could be non-negligible, it may partially mask or amplify the effect of a variation in K_5 on the width of a pH activity profile. In Fig. 6*A*, the variation of $\Delta\Delta pK$ with ion pair stability (*i.e.* $-\log(K_{5mut}/K_{5WT})$) is simulated for values of $(\Delta pK_4 - \Delta pK_3)$ ranging from −1 to +1. It is evident from Fig. 6*A* that the value of K_{5mut} determined from $\Delta\Delta pK$ is strongly dependent on $(\Delta pK_4 - \Delta pK_3)$. It can be seen also that the narrowing of the pH activity profile reaches a maximum when the ion pair is destabilized approximately 100-fold or more (i.e. $-\log(K_{5mut}/K_{5WT}) \geq 2$). Further reduction in ion pair stability does not lead to additional narrowing of the profile, and when the pH activity profile of a mutant enzyme reaches this theoretical maximum value of $\Delta\Delta pK$, it is only possible to put a higher limit to the value of K_{5mut}. However, the relationship between $(k_{cat}/K_M)^{lim}$ and K_5 is linear when the ion pair is significantly destabilized (Fig. 6*B*), indicating that perturbation of the ion pair will contribute to a decrease in $(k_{cat}/K_M)^{lim}$ even past the limit where no further effect on pH activity profiles is discernible. Therefore, caution has to be used when interpreting results of pH activity measurements in terms of perturbation of the stability of the ion pair form of the active site residues.

It is important to dissect out the contribution of ion pair stability to the measured kinetic parameters when trying to elucidate the role of Asn^{175} in the catalytic mechanism of papain. From the above considerations, it is obvious that this cannot be accomplished in a straightforward manner to yield a definitive answer. However, the data can be interpreted to define limits to the contribution of Asn^{175} to various aspects of the catalytic mechanism. With the Asn^{175} → Gln mutant, the hydrogen bond between the side chains of residues 175 and 159 is believed to be maintained (see below), and in a first approximation we can consider that $(\Delta pK_4 - \Delta pK_3) = 0$ for this mutant. Since $\Delta\Delta pK = -1.07$ for Asn^{175} → Gln (Table I), we can calculate that $K_{5mut} = 0.58$ and replacement of Asn^{175} by a glutamine causes a 7.6-fold destabilization of the ion pair form

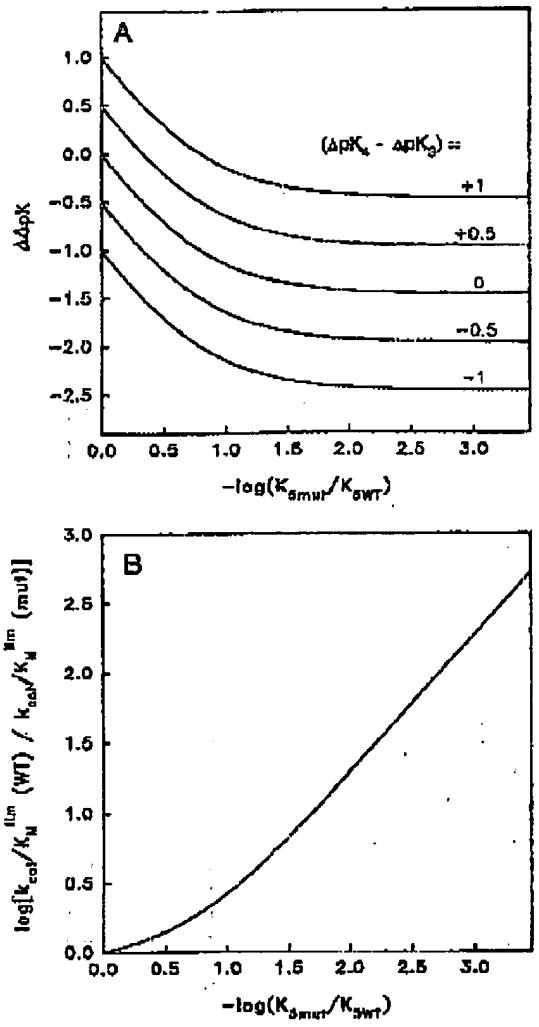

FIG. 6. **Simulated curves illustrating the relationships between kinetic parameters and ion pair stability.** *Panel A*, relationship between the variation in width of a pH activity profile ($\Delta\Delta pK$) and the perturbation of ion pair stability for various values of ($\Delta pK_a - \Delta pK_b$). *Panel B*, relationship between the variation in the experimentally determined activity (*i.e.* limiting k_{cat}/K_M value obtained from pH activity profiles) and the perturbation of ion pair stability.

compared to wild-type papain. Knowing the effect of the mutation on the ion pair stability, we can now calculate the effect on the intrinsic activity of the enzyme. To do this, we can use the previously determined equation (Equation 3),

$$(k_{cat}/K_M)^{lim}_{mut} = (k_{cat}/K_M)_{WT} \cdot f \cdot \left(\frac{K_{\delta mut}}{K_{\delta mut} + 1}\right) \qquad \text{(Eq. 8)}$$

where f represents the effect of a mutation on the intrinsic activity of the enzyme (*i.e.* the ratio of the intrinsic k_{cat}/K_M for mutant over wild-type papain), $(k_{cat}/K_M)_{WT}$ is the intrinsic value of k_{cat}/K_M for wild-type papain, and $(K_{\delta mut}/(K_{\delta mut} + 1))$ reflects how the perturbation of K_δ resulting from a mutation will affect the measured specificity constant (27). For Asn^{175} → Gln, $f = 0.67$ (Table II), indicating that the intrinsic activity of the mutant is only 1.5-fold lower than that of wild-type papain.

To account for the possibility that ($\Delta pK_a - \Delta pK_b$) might not be negligible for the Asn^{175} → Gln mutant, limits can be put on

TABLE II

Contribution of the Asn^{175} mutations to ion pair stability and intrinsic activity

Enzyme	K_δ	$K_\delta/K_{\delta mut}$	f	($\Delta pK_a - \Delta pK_b$)
Wild-type	4.4	2200		
Asn^{175} → Gln[a]	0.58	290	0.67	0
Asn^{175} → Gln (limiting case 1)[b]	4.4	2200	0.20	−1.07
Asn^{175} → Gln (limiting case 2)[c]	0.33	165	1	+0.15
Asn^{175} → Ala (limiting case 1)[b]	4.4	2200	0.0072	−1.58
Asn^{175} → Ala (limiting case 2)[c]	0.0060	3.0	1	−0.12

[a] Calculations done assuming that ($\Delta pK_a - \Delta pK_b$) = 0 for the Asn^{175} → Gln mutant.

[b] Calculations done assuming that the Asn^{175} → Gln or Ala mutation has no effect on ion pair stability, *i.e.* $K_{\delta mut} = 4.4$.

[c] Calculations done assuming that the Asn^{175} → Gln or Ala mutation has no effect on the intrinsic activity, *i.e.* $f = 1$.

the contribution of Asn^{175} to the catalytic mechanism by considering that ion pair destabilization is responsible for none or all of the observed variation in activity. In the first case where the stability of the ion pair is considered not to be affected by the mutation, $K_{\delta mut} = 4.4$ and the decrease in activity is due in totality to a decrease in intrinsic activity of the enzyme. With $\Delta\Delta pK = -1.07$, we can calculate ($\Delta pK_a - \Delta pK_b$) and f using Equations 2 and 3, respectively. In the second limiting case, we consider that all of the effect on the experimentally determined specificity constant originates from a perturbation of the ion pair and that mutation of Asn^{175} to Gln has no influence on the intrinsic activity of the enzyme, *i.e.* $f = 1$. The results using both assumptions, given in Table II, place reasonable limits on the magnitude of the effect that mutation of Asn^{175} to a glutamine can have on the intrinsic activity and on the ion pair stability of papain (assuming of course that the Asn^{175} → Gln mutation does not stabilize the thiolate-imidazolium ion pair or increase the intrinsic activity of the enzyme). It can be seen in Table II that the conclusions are similar to when the variation in ($\Delta pK_a - \Delta pK_b$) was considered negligible, i.e. the mutation has only a small effect on ion pair stability and/or intrinsic activity.

For the Asn^{175} → Ala mutant, only the two limiting cases were considered since the probability that the value of ($\Delta pK_a - \Delta pK_b$) is affected by the mutation is much higher. In the first limiting case (no effect on ion pair stability), the value of f = 0.0072 indicates that the intrinsic activity of the mutant Asn^{175} → Ala is lower than that of papain by a factor of no more than 140. By considering that the mutation has no effect on intrinsic activity (case 2), $K_{\delta mut} = 0.0060$, a value 735 times lower than that of wild-type papain, which places an upper limit to the ion pair destabilization upon mutation of Asn^{175} to Ala.

DISCUSSION

The role of the asparagine residue in the Cys-His-Asn "catalytic triad" of cysteine proteases has been investigated by replacing Asn^{175} in papain with an alanine or a glutamine residue by site-directed mutagenesis. The kinetic data obtained with the substrate Cbz-Phe-Arg-MCA indicate that Asn^{175} can be replaced by a Gln residue without major changes in the specificity constant $(k_{cat}/K_M)^{pbs}$, while mutation to an Ala residue leads to a 150-fold decrease in activity. The side chain of a glutamine retains the possibility of forming a hydrogen bond with the side chain of His^{159} and the higher activity of the Asn^{175} → Gln mutant compared to Asn^{175} → Ala could be explained by the existence of such a hydrogen bond. Computer modeling indeed suggests that the hydrogen bond distances between the side chain amide of residue 175 and His^{159} in

wild-type papain can be maintained in the Asn^{175} → Gln mutant. When the possibility of residue 175 forming such a hydrogen bond to His^{159} is removed, *i.e.* by mutating Asn^{175} to an alanine, the catalytic efficiency is reduced by about 2 orders of magnitude. However, the Asn^{175} → Ala mutant still hydrolyzes the substrate Cbz-Phe-Arg-MCA at a rate much higher than the non-catalytic rate; therefore, Asn^{175} cannot be considered as an essential catalytic residue in the cysteine protease papain.

A significant fraction of papain exists with the Cys^{25} and His^{159} residues as an ion pair at neutral pH, and from theoretical considerations Asn^{175} has been proposed to stabilize the thiolate-imidazolium ion pair at the active site of papain (19). For a linear peptide containing non-interacting cysteine and histidine residues, if K_{CH} is used to designate the ratio of the concentration of the peptide where both side chains are ionized to the concentration where both side chains are neutral, then it can be shown that $\log(K_{CH}) = (pK_a \text{ His} - pK_a \text{ Cys})$. By using 9.1 and 6.4 for the pK_a values of cysteine and histidine, respectively (28), we obtain a value of 0.0020 for K_{CH}. The value of the corresponding equilibrium constant in papain between the ion pair form and the neutral form of the active site residues has been estimated at 4.4 (27). Therefore, in wild-type papain the ion pair is approximately 2200-fold more stable than if the Cys and His residues were non-interacting in a linear peptide (K_S/K_{CH} in Table II). The mutation of Asn^{175} to Gln is accompanied by an 8-fold decrease in the stability of the thiolate-imidazolium ion pair. Once the ion pair is formed, there is virtually no difference in activity between Asn^{175} → Gln and wild-type papain ($f = 0.67$), suggesting that the advantage of having an Asn at position 175 over a Gln is mainly to stabilize the ion pair. If limiting cases are considered, the value of K_S can decrease by up to 13-fold while the effect on intrinsic activity is of no more than 3-fold ($f = 0.30$). For the Asn^{175} → Ala mutant, a major perturbation (narrowing) of the pH activity profile is observed and the kinetic data can only be used to put limits to the magnitude of the effects on intrinsic activity and ion pair stability. For example, if the replacement of Asn^{175} by an alanine has a negligible effect on the intrinsic activity of the enzyme (case 2 in Table II), the ion pair stability would be decreased by 735-fold ($K_S = 0.0060$ compared to 4.4 for wild-type papain), to a value of K_S that is only 3 times that of K_{CH} for non-interacting residues in a linear peptide. For case 1, where the decrease in observed activity is suggested to be entirely due to a decrease in intrinsic activity, the mutation would have no effect on ion pair stability. It must be noted, however, that in limiting case 1, a relatively high value of $(\Delta pK_1 - \Delta pK_2) = -1.53$ is needed to account for the experimental data. It is most likely that the variations in kinetic parameters observed for the Asn^{175} → Ala mutation are the result of a combination of effects on ion pair stability and intrinsic activity, *i.e.* intermediate between cases 1 and 2.

As discussed above, it is difficult to dissect out the relative effect of the mutation on ion pair stability and intrinsic activity for the Asn^{175} → Ala mutant. However, it is interesting to note that, according to the model linking ion pair stability to pH activity profiles (27), we would expect a strong perturbation in ion pair stability to be accompanied by an important narrowing of the pH activity profile. The quantitative interpretation of pH activity data depends, however, on the correct assignment of pK_a values to active site groups of the enzyme. Recently, the possibility that the increase in k_{cat}/K_M at low pH shown in Fig. 4 could be the result of ionizations other than that of Cys^{25} in the papain molecule was raised (29). If this is the case, the pK_a values measured would not reflect ion pair formation. Changes in the pH activity profile would be the result of variations in reactivity of different protonic forms of the enzyme, without variations in the pK_a values of the groups that modulate activity. This model is relatively complex and requires a large number of parameters to describe the pH activity profiles. Even though this possibility cannot be ruled out unequivocally, we believe that ion pair destabilization leading to narrowing of pH activity profiles is the most likely explanation for our results. The fact that both the acid limb and basic limb pK_a values are affected by the mutations provides a strong argument in favor of a perturbation of ion pair stability. A probable (but not necessary) consequence of ion pair destabilization is that both the acid and basic limbs will be affected. Even though the assignment of the ionization of Cys^{25} to either one of the two pK_a values observed in the acid limb of the pH activity profile for the wild-type enzyme cannot be made unambiguously, as concluded previously (27), the data presented for mutants of Asp^{158} by Ménard *et al.* (27) and for mutants of Asn^{175} (this paper) can all be rationally explained by considering electrostatic effects and ion pair perturbation on a relatively simple model considering in a first approximation only one active form of the enzyme and three ionizable groups. Although more complex explanations cannot be ruled out, we continue to favor the simplest model that fully accounts for the experimental results presented in this report and all related reports originating from this laboratory. Experiments are in progress, however, to clarify this point.

The geometry of the active site Cys-His-Asn residues in cysteine proteases is very similar to that of the corresponding Ser-His-Asp residues forming the catalytic triad of serine proteases (30). Replacement of the Asn^{175} side chain in papain by that of an Ala residue is evaluated to decrease the intrinsic activity of the enzyme by a factor of no more than 140. For serine proteases, mutation of the Asp residue to Ala (for subtilisin) and Ser (for trypsin) resulted in approximately 10^4-fold reductions in enzymatic activity (31, 32). However, an Asp^{102} → Asn mutant of trypsin displayed only 10^2- to 10^3-fold decreases in activity, depending on the nature of the leaving group of the substrate (33). This latter mutation is peculiar in that the side chain can form a hydrogen bond with the active site His residue (34). In addition, it has been shown that even though the presence of the negative charge adjacent to His^{57} in trypsin is important for activity, its precise location is not critical. Indeed, an alternate geometry for the catalytic triad of serine proteases has been proposed (32). The latter two results indicate that certain modifications of the catalytic triad in serine proteases are tolerated.

The magnitude of the apparent contribution to enzymatic activity of the Asn residue in cysteine proteases and the corresponding Asp residue in serine proteases may reflect basic differences in the catalytic mechanism of the two classes of enzymes (35). In the case of serine proteases, the formation of the transition state and tetrahedral intermediate is accompanied by charge separation, and it has been suggested that the negative charge on the aspartate can help this process through electrostatic stabilization, therefore contributing to catalysis (36). In cysteine proteases, the Asn residue in the catalytic triad might be of importance for stabilizing the ion pair form of the catalytic residues (*i.e.* the ground state of the enzyme) by contributing to maintain the active site residues in a favorable geometry. In contrast to serine proteases, charge separation is already present in the ground state and generation of the transition state and tetrahedral intermediate causes only a rearrangement of the charges. In addition, the Asn residue could play a role in catalysis through the orientational effect of the H bond to His^{159}. This hydrogen bond allows rotation of the His^{159} side chain to orient the imidazole group in a proper position to act as a proton donor to the leaving group of the

substrate. The fact that a C-S bond is weaker than a C-O bond and that the thiolate anion is a very good leaving group can explain the necessity of such a step in cysteine proteases. Therefore, the full catalytic power of the triad might be better exploited in the hydrolysis of non-activated peptide bonds, whereas the activity of the Asn[175] mutants of papain was measured against a small activated peptidyl substrate. For this reason, the influence of the mutations on activity against protein substrates might be more important than the measured effects with the substrate Cbz-Phe-Arg-MCA, but the very low amount of enzyme available from the expression system precludes such studies. It must be noted also that preliminary results with cathepsin S (data not shown) show that mutation of Asn[175] has a stronger effect on activity than that observed for papain, indicating that the magnitude of the Asn[175] contribution to enzymatic activity might differ from one cysteine protease to another.

Highly conserved residues at the active site of enzymes are often regarded as being essential for activity. For the cysteine proteases, it is difficult to account for the strict conservation of Asn[175] based exclusively upon enzymatic activity, given the relatively modest effect of amino acid substitution at position 175. Indeed, our results show that presence of a Gln at position 175 is almost neutral with respect to the enzyme activity. However, within a large data base of cysteine protease sequences, no residue is found other than an asparagine (37). The strict conservation of Asn[175] might therefore be the consequence of properties in addition to the catalytic activity of the enzyme. Wild-type propapain accumulates in the yeast cell vacuole (20) mostly as a soluble, protease-resistant species. Replacement of Asn[175] by either a Gln or Ala increased the fraction of insoluble, protease-susceptible propapain, suggesting that these mutations alter the ability of the protein to fold into a functional protease precursor. In addition, the mature papain mutants resulting from the processing of the properly folded proenzymes have an increased rate of thermal inactivation, indicating that the mutations affect the thermal stability of the mature enzyme. The acetamide H atoms of Asn[175] in wild-type papain interact with the aromatic rings of Trp[177] and Trp[181], and perturbation of these interactions in the Asn[175] → Gln mutant could contribute to the decrease in stability of the enzyme. The computer modeling experiments indicate that the Gln[175]-His[159] hydrogen bond can be formed with or without perturbation of the interactions with the Trp residues and, therefore, cannot unambiguously support or refute this hypothesis. A similar structural role has been established recently for the catalytic histidine residue at the active site of phospholipase A_2 (38). Our results indicate that in addition to its contribution to the catalytic properties of the enzyme, Asn[175] participates in the folding pathway (39) and in the thermal stability of the folded protein. The Asn[175] residue in cysteine proteases could constitute another example of the conservation of an active site residue resulting from a combination of functional and structural constraints.

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